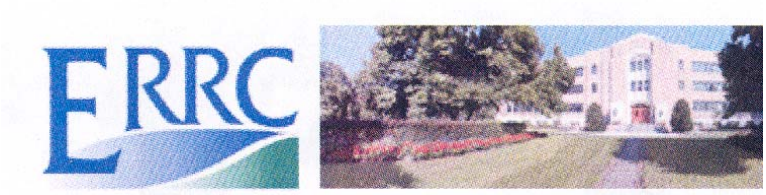

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Research Note

Reduction of *Escherichia coli* O157:H7 and *Salmonella* spp. on Laboratory-Inoculated Mung Bean Seed by Chlorine Treatment†

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ABSTRACT

Three U.S. outbreaks of foodborne illness due to consumption of contaminated raw mung bean sprouts occurred in the past 2 years and were caused by *Salmonella* Enteritidis. The original source of the pathogens is thought to have been the seed. The aim of this study was to determine whether treatment with aqueous chlorine would eliminate the pathogens from mung bean seed inoculated in the laboratory with four-strain cocktails of *Escherichia coli* O157:H7 and *Salmonella* spp. Treatments (for 5, 10, or 15 min) with buffered (500 mM potassium phosphate, pH 6.8) or unbuffered solutions containing 0.3 or 3.0% (wt/vol) $Ca(OCl)_2$ were tested. In order to mimic common commercial practice, seed was rinsed before and after treatment with sterile tap water. Treatment for 15 min with buffer (500 mM potassium phosphate, pH 6.8) or sterile water in combination with the seed rinses resulted in maximum reductions of approximately 3 $\log_{10}$ CFU/g. The largest reductions (4 to 5 $\log_{10}$ CFU/g) for the chlorine treatments in combination with the rinses were obtained after treatment with buffered 3.0% (wt/vol) $Ca(OCl)_2$ for 15 min. Treatment of mung bean seed for 15 min with unbuffered or buffered 3.0% (wt/vol) $Ca(OCl)_2$ did not adversely affect germination. Even though treatments with 3% (wt/vol) $Ca(OCl)_2$ in combination with the water rinses were effective in greatly reducing the populations of both bacterial pathogens, these treatments did not result in the elimination of the pathogens from laboratory-inoculated seed.

Alfalfa and other types of sprouts that are often consumed raw are designated a special food safety problem by the National Advisory Committee on Microbiological Criteria for Foods *(14)* because of the ability of human pathogens such as *Salmonella* spp. and *Escherichia coli* O157:H7 to rapidly multiply on sprouts during propagation and because of the lack of a postharvest kill step in the processing of these sprouts. Since 1995 in the United States there have been numerous outbreaks of salmonellosis and two outbreaks caused by *E. coli* O157 (O157:H7 and O157:NM) due to the consumption of contaminated alfalfa and clover sprouts *(14, 20)*. In almost all outbreaks, on the basis of direct isolation and/or epidemiological evidence, the initial source of the contaminant was believed to be the seed *(14)*. The first reported foodborne outbreak (45 cases) in the United States due to the consumption of raw mung bean sprouts occurred in the spring of 2000 in California (press release no. 22-00, 19 August 2000, http://www.dhs.ca.gov) and was due to contamination with *Salmonella* Enteritidis. Since then, two additional mung bean sprout–related outbreaks of salmonellosis have occurred in the United States *(17, 18)*. Outbreaks of salmonellosis attributable to mung bean sprouts had previously been reported in countries other than the United States, including an outbreak in the United Kingdom that resulted in 143 cases *(15)*. In the investigation of this outbreak, *Salmonella* spp. were isolated both from sprouts and seed.

Salmonella spp. can grow rapidly on mung beans during the germination process. In a study involving laboratory-inoculated mung bean seed, a mixture of *Salmonella* Anatum and *Salmonella* Montevideo was demonstrated to increase from initial populations of 0.41 and 2.52 $\log_{10}$ CFU/g of seed to populations of 5.23 and 6.75 $\log_{10}$ CFU/g of sprouts, respectively, over a 2-day sprouting period *(1)*.

Several reports on the efficacy of chlorine in the reduction of *Salmonella* spp. and *E. coli* O157:H7 on laboratory-inoculated alfalfa seed have appeared *(10, 11, 19, 24)*. Two recent U.S. Food and Drug Administration guidance documents *(21, 22)* and a video released jointly by the U.S. Food and Drug Administration and the State of California Department of Health Services *(23)* have recommended the treatment of alfalfa and other sprouting seed with 20,000 ppm of free chlorine from $Ca(OCl)_2$ (or with an equivalent antibacterial treatment) for 15 min and the testing of spent irrigation water for the presence of *Salmonella* spp. and *E. coli* O157:H7. However, there are no published studies on the efficacy of chlorine treatment of sprouting seeds other than alfalfa seed in the elimination of human pathogens. Because of the recent foodborne outbreaks involving mung bean sprouts and the lack of published information on the effectiveness of chlorine treat-

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† Mention of a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

ments on this sprouting seed type, this study was conducted to determine the efficacy of the treatment of laboratory-inoculated mung bean seed with chlorine provided by $Ca(OCl)_2$ in combination with seed rinses with water with regard to the elimination of *E. coli* O157:H7 and *Salmonella* spp.

MATERIALS AND METHODS

Bacterial strains and seed. Strains of *E. coli* O157:H7 used in this study were strains F4546 (a clinical strain associated with a sprout-related outbreak that occurred in Michigan and Virginia in 1997), SEA13B88 (isolated from apple cider implicated in an outbreak in the western United States in 1996), C7927 (a clinical strain associated with an apple cider–related outbreak in Massachusetts in 1992), and Ent-C9490 (a clinical strain associated with a ground beef–related outbreak in the western United States in 1993). All strains except for F4546 were obtained from Dr. Pina Fratamico (U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center). Strain F4546 was obtained from Dr. Robert Buchanan (U.S. Food and Drug Administration). *Salmonella* Anatum F4317, *Salmonella* Infantis F4319, *Salmonella* Newport H1275, and *Salmonella* Stanley HO558 were obtained from Dr. Patricia Griffith (Center for Disease Control and Prevention, Atlanta, Ga.). All four strains were implicated in sprout-related outbreaks in the United States. No inhibition among the four bacterial strains of *Salmonella* or *E. coli* O157:H7 was noted when the strains were streaked perpendicular to each other on tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.) and incubated for 24 h at 37°C. For long-term storage, strains were stored at −80°C in tryptic soy broth (TSB; Difco) plus 20% glycerol.

Mung bean seed was purchased from International Specialty Supply (Cookeville, Tenn.). The native microflora was eliminated by exposing the seed contained in stomacher bags with mesh liners to 25 kGy of gamma irradiation. Sterility was confirmed by enrichment. Irradiated seed (10 g) was placed into a sterile stomacher bag, 40 ml of TSB was added, and the bag's contents were incubated at 30°C for 20 h. After incubation, four 0.25-ml samples were plated onto TSA plates. Plates were incubated at 30°C for 48 h and then examined for growth.

Seed inoculation. To prepare inoculum, bacterial strains were streaked from frozen stock cultures onto TSA, and plates were incubated for 18 h at 37°C. A single colony was used to inoculate TSB (3 ml; one tube per strain). The broth cultures were incubated for 18 h in a shaking incubator (250 rpm) at 37°C. For *E. coli* O157:H7, starter cultures (0.1 ml) were used to inoculate 25 ml of TSB with 1.0% (wt/vol) dextrose (Difco) (with an additional 7.5 g/liter) to induce pH-dependent stationary-phase acid resistance *(3, 4)*. The broth media were contained in 125-ml flasks (two flasks per strain) and incubated statically at 37°C for 18 h. The same procedure was used for *Salmonella* except that TSB was not supplemented with additional dextrose and the broth cultures were shaken at 250 rpm. After incubation, the contents of the eight flasks were combined in a single sterile 500-ml flask and mixed, and then the mixture of strains was transferred to sterile centrifuge bottles. Bacterial cells were pelleted by centrifugation, the pellets were washed once with sterile 0.1% (wt/vol) peptone water (PW; Difco), the washed cells were recentrifuged, and the final cell pellets were resuspended in 250 ml of PW. The cell concentration in the final inoculum was determined by preparing decimal dilutions in PW and plating in duplicate onto TSA (0.1 ml per plate). Colonies were counted after incubation for 18 h at 37°C. For the inoculation of mung bean seed, 50 ml of the inoculum cocktail was added to each of five stomacher bags containing 100 g of sterile mung bean seed per bag. The bags were massaged by hand for 1 min, and excess inoculum was decanted. The mesh liners containing the inoculated seed were placed under a biosafety cabinet on a sterile tray covered with wire mesh and allowed to dry for 24 h. To determine the initial inoculum load on the seed, 20 ml of buffered PW was added to a sterile blender receptacle containing 10 g of inoculated seed. The seed was homogenized for 1 min with a commercial blender, and then decimal dilutions of the homogenate were prepared with PW. For *E. coli* O157:H7, dilutions were plated in triplicate onto TSA and eosin methylene blue agar (Difco) (0.1 ml per plate), as well as Petrifilm *E. coli*/coliform and *Enterobacteriaceae* count plates (1.0 ml per plate; 3M). For the *Salmonella* strains, dilutions were plated in triplicate onto TSA and XLT-4 (Difco). Colonies were counted after 24 and 48 h of incubation at 37°C. Dried inoculated seed was placed into sterile stomacher bags and stored at 4°C until it was used. Experiments were completed within 2 months of the time of seed inoculation. No change in the pathogen population on the inoculated seed was noted.

Seed treatments. Sanitizer solutions consisted of 0.3 and 3.0% (wt/vol) calcium hypochlorite (65% active chlorine; Aldrich Chemical Company, Milwaukee, Wis.) prepared in room temperature sterile tap water (the final pHs of the solutions were 10.4 and 11.6, respectively) or 500 mM potassium phosphate buffer (pH 6.8) (final pH of 6.8). Sanitizer solutions were stirred for 20 min at room temperature before they were used, and free chlorine content was determined after appropriate dilution with purified water (18.2 MΩ-cm resistivity) with an EPA approved commercial test kit (Accuvac, Hach Company, Loveland, Colo.) based on the *N,N*-diethyl-*p*-phenylenediamine method. All glassware used in the preparation of dilutions was treated with diluted (1:1,000) commercial chlorine bleach, rinsed, and dried in an oven to remove chlorine-binding sites before it was used. Ten grams of inoculated seed was placed into sterile stomacher bags, and the seed was rinsed two times by the addition of 50 ml of sterile tap water followed by agitation by hand for 2 min for each wash. Fifty milliliters of the sanitizer solution (or sterile tap water or buffer, as appropriate for the controls) was then added, and the bag contents were agitated by hand for 5, 10, or 15 min. After the sanitizer solution was decanted, the treated seed was rinsed two times with sterile tap water as described above. After the final rinse, 20 ml of D/E neutralizing broth (Difco) was added, and the mixture was transferred to a sterile blender receptacle. The sample was homogenized for 10 s with a commercial blender. As a further control, unrinsed and untreated seed was homogenized. Serial decimal dilutions were prepared from the homogenates in sterile PW, and serial dilutions and undiluted homogenate were plated in triplicate on Petrifilm *E. coli*/coliform count plates (1.0 ml per plate) and TSA (0.1 ml per plate) for *E. coli* O157:H7 and on XLT-4 and TSA (0.1 ml per plate) for the *Salmonella* strains. Plates were incubated at 37°C, and final CFU counts were recorded after 24 to 48 h. Two random colonies per plate of TSA were confirmed to be *E. coli* O157 or *Salmonella* spp. by a commercial latex agglutination test (RIM, Remel, Lenexa, Kans.) or by slide agglutination using *Salmonella* O antiserum Poly AI and Vi (Difco), respectively.

Seed germination tests. Nonirradiated seed samples consisting of 100 seeds each (6.5 to 6.7 g) were placed in 100-ml glass beakers. Seeds were rinsed twice with 50 ml of sterile tap water for 2 min with mechanical stirring. Next, 50 ml of 0.3 or 3.0% (wt/vol) calcium hypochlorite solution prepared in sterile tap water or 500 mM KP buffer (pH 6.8) (1,800 and 16,000 ppm free

TABLE 1. *Effect of chlorine treatment on* E. coli O157:H7 *inoculated onto mung bean seed*

Treatment[a]	pH	Time (min)	Log reduction (CFU/g) on[b]: TSA	Petrifilm
Sterile buffer[c]	6.8	5	1.88 BC	1.59 CD
		10	1.97 BC	2.17 BCD
		15	3.23 AB	3.51 AB
Sterile tap water	8.5	5	1.30 C	1.30 D
		10	2.22 BC	1.75 BCD
		15	2.73 ABC	2.52 ABCD
$Ca(OCl)_2$, 3.0% (wt/vol) in water	11.6	5	1.94 BC	1.58 CD
		10	2.75 ABC	2.33 ABCD
		15	2.73 ABC	2.31 ABCD
$Ca(OCl)_2$, 0.3% (wt/vol) in buffer	6.8	5	2.52 ABC	2.36 ABCD
		10	3.24 AB	3.30 ABC
		15	3.25 AB	2.84 ABCD
$Ca(OCl)_2$, 3.0% (wt/vol) in buffer	6.8	5	2.54 ABC	2.53 ABCD
		10	3.22 AB	3.33 ABC
		15	3.93 A	4.03 A

[a] Seed was rinsed twice with sterile water before and after each treatment.
[b] Values shown are the means of data from three separate trials. Means with the same letters in the same column are not significantly different ($P < 0.05$).
[c] 500 mM potassium phosphate, pH 6.8.

chlorine, respectively) was added, and the suspension was stirred mechanically for 15 min. After the chlorine solutions were decanted, seeds were again rinsed twice with sterile water as described above. Seeds were then transferred to sterile glass culture dishes lined with wetted filter paper and left to germinate on a lab bench at room temperature for 2 days. Seeds with emerging roots visible to the naked eye were considered germinated. The germination experiments were repeated once.

Statistical analysis. Data were analyzed for significant differences ($P < 0.05$) by the least-significant-difference separation procedure *(13)*.

RESULTS AND DISCUSSION

The irradiated mung bean seed was deemed sterile on the basis of the lack of microbial growth after broth enrichment and plating to TSA. The use of sterile seed allowed us to easily determine the number of injured bacterial cells present by comparing plate counts for nonselective media with those for selective media. Inoculum cocktails containing large populations of each pathogen (9.00 $\log_{10}$ CFU/ml for *E. coli* O157:H7 and 9.97 $\log_{10}$ CFU/ml for the *Salmonella* strains) were used to obtain seeds with a

TABLE 2. *Effect of chlorine treatment on* Salmonella *strains inoculated onto mung bean seed*

Treatment[a]	pH	Time (min)	Log reduction (CFU/g) on[b]: TSA	XLT-4
Sterile buffer[c]	6.8	5	2.88 B	2.21 BC
		10	2.78 B	2.84 ABC
		15	3.58 AB	3.08 ABC
Sterile tap water	8.5	5	2.73 B	1.59 C
		10	2.70 B	2.56 ABC
		15	3.55 AB	2.54 ABC
$Ca(OCl)_2$, 3.0% (wt/vol) in water	11.6	5	3.71 AB	3.45 ABC
		10	3.87 AB	3.45 ABC
		15	4.26 AB	3.94 AB
$Ca(OCl)_2$, 0.3% (wt/vol) in buffer	6.8	5	3.30 AB	2.65 ABC
		10	3.94 AB	3.34 ABC
		15	3.80 AB	3.31 ABC
$Ca(OCl)_2$, 3.0% (wt/vol) in buffer	6.8	5	4.44 AB	4.19 AB
		10	3.82 AB	3.42 ABC
		15	5.02 A	4.65 A

[a] Seed was rinsed twice with sterile water before and after each treatment.
[b] Values shown are the means of data from three separate trials. Means with the same letter in the same column are not significantly different ($P < 0.05$).
[c] 500 mM potassium phosphate, pH 6.8.

TABLE 3. *Effect of chlorine treatment (15 min) on germination of mung bean seed*

Treatment	pH	% Germination on[a]: Day 1 Trial A	Day 1 Trial B	Day 2 Trial A	Day 2 Trial B
Sterile tap water	8.5	100	99	100	99
Sterile buffer[b]	6.8	97	91	97	100
$Ca(OCl)_2$, 0.3% (wt/vol) in:					
Tap water	10.4	100	100	100	100
Buffer	6.8	92	95	98	99
$Ca(OCl)_2$, 3.0% (wt/vol) in:					
Tap water	11.6	98	98	99	100
Buffer	6.8	94	100	99	100

[a] For each trial, 100 seeds per treatment were germinated.
[b] 500 mM potassium phosphate, pH 6.8.

high level of inoculum. Ideally, the inoculum load should be ≥ 5 $\log_{10}$ CFU/g to demonstrate a 5-log reduction. Cells of *E. coli* O157:H7 induced to pH-dependent stationary-phase acid resistance *(3, 4)* used as bacteria in the field may be exposed to a variety of environmental stresses, including dehydration and nutrient depravation. Similar acid-adapted cells of the *Salmonella* strains were not used for inoculation because acid-adapted cells of *Salmonella* Typhimurium were reported to have increased sensitivity to hypochlorous acid *(12)*. For *E. coli* O157:H7, the results from plating on the four media (three selective and one nonselective) were similar, indicating an initial inoculum load of 4.1 to 4.2 $\log_{10}$ CFU/g on the seed before storage at 4°C. For the *Salmonella* strains, plating on the nonselective agar medium (TSA) indicated that inoculated seed harbored a population of 7.6 $\log_{10}$ CFU/g, while plating on the selective medium (XLT-4) indicated an initial population of 6.8 $\log_{10}$ CFU/g. As evidenced by the similar counts obtained for selective and nonselective media, the inoculation and drying procedures did not lead to substantial bacterial cell injury for the two pathogens.

In this study, seed was rinsed with sterile tap water both before and after chlorine treatments to correspond with U.S. Food and Drug Administration and State of California grower recommendations *(23)*. A very high concentration (500 mM) of buffer was required to maintain a pH of 6.8 for the chlorine solutions containing 3.0% (wt/vol) $Ca(OCl)_2$. The unbuffered 0.3% (wt/vol) $Ca(OCl)_2$ solutions (final pH of 8.9) provided 1,900 ppm free chlorine, while unbuffered 3.0% (wt/vol) $Ca(OCl)_2$ (final pH of 11.6) provided 18,000 ppm free chlorine. Buffered 0.3 and 3.0% (wt/vol) $Ca(OCl)_2$ (final pH of 6.8) provided 1,800 and 16,000 ppm free chlorine, respectively. At a pH of 6.8 and a temperature of 20°C, approximately 75% of the chlorine in the solution is in the form of hypochlorous acid, while at a pH of 11.0 at 20°C, only 0.03% is in this form, with the remainder being in the form of the hypochlorite ion *(7)*. Hypochlorous acid is thought to be the primary antimicrobial agent in chlorine solutions and is reported to have approximately 80 times as much antibacterial activity as the hypochlorite ion *(7)*. The high alkalinity of the unbuffered solutions containing 18,000 ppm free chlorine may contribute to their efficacy. Weissinger and Beuchat *(24)* reported that 1% $Ca(OH)_2$ and 1% calcinated calcium (both with pHs above 12) were as effective as 20,000 ppm free chlorine in reducing populations of *Salmonella* spp. on laboratory-inoculated alfalfa seed.

For seed inoculated with *E. coli* O157:H7 or the *Salmonella* strains, treatment with sterile tap water or buffer alone in combination with the water rinses before and after treatment led to substantial reductions in bacterial populations (up to 3.6 $\log_{10}$ CFU/g) (Tables 1 and 2). For *E. coli* O157:H7, the largest reduction (3.9 $\log_{10}$ CFU/g) was obtained after a 15-min treatment with buffered 3.0% (wt/vol) $Ca(OCl)_2$ along with the seed rinses (Table 1). This was also true for the *Salmonella* strains (with a log reduction of 5.02; Table 2). Even though these log reductions were larger than those observed for the corresponding buffer controls, statistical analyses indicated that the differences were not significant (Tables 1 and 2). Chlorine treatments were more effective in the presence of buffer. In general, a longer treatment time led to a larger log reduction. On the basis of the log reduction data obtained for selective agar media, there was no indication of large populations of injured cells for *E. coli* O157:H7 or the *Salmonella* strains after chlorine treatment. Germination of mung bean seed treated with 0.3 or 3.0% (wt/vol) calcium hypochlorite prepared in sterile tap water or buffer for 15 min did not differ from that of the control seed over a 48-h germination period (Table 3).

The log reductions observed for the *Salmonella* strains after sterile water, buffer, or chlorine treatments were larger than those observed for *E. coli* O157:H7 on seed treated with the same concentrations (Tables 1 and 2). This finding may be a reflection of the larger initial bacteria load for the *Salmonella* strains than for *E. coli* O157:H7 on the seed, because both pathogens are highly susceptible to killing with chlorine when cells are present in suspension *(5, 16)*. It is also possible that a larger percentage of the cells of *E. coli* O157:H7 present on the seed may be located in areas that are difficult for the sanitizer to reach (e.g., the hilum, the micropyle, cracks in the seed coat) or that cells of this pathogen attach more tightly to the seed surface. Another

contributing factor may be that the *E. coli* O157:H7 cells used to prepare the inoculum cocktail were induced to pH-dependent stationary-phase acid resistance. Such cells exhibit cross-protection from other stresses *(3)* and may be more resistant to chlorine.

The inability of aqueous solutions containing high levels of free chlorine to totally eliminate *E. coli* O157:H7 and the *Salmonella* strains from laboratory-inoculated mung bean seed is consistent with the results of similar studies involving alfalfa seed inoculated in the laboratory with *Salmonella* spp. or *E. coli* O157:H7 *(2, 8, 11, 19, 24).* This finding may indicate that when sprouting seed is inoculated through soaking in an aqueous bacterial suspension in the laboratory and subsequently dried, the bacteria are located in areas that are not readily accessible to aqueous solutions of antimicrobial compounds. This may or may not be true for naturally contaminated mung bean seed. For laboratory-inoculated mung bean seed, antimicrobial compounds delivered in the gas phase appear to be more effective in reaching bacteria in protected locations on seeds than aqueous treatments do. Delaquis et al. *(6)* reported that treatment of inoculated mung bean seed with gaseous acetic acid (242 μl/liter of air for 12 h at 45°C) eliminated *Salmonella* Typhimurium and *E. coli* O157:H7. The treatment of inoculated mung bean seed with ammonia (180 or 300 mg/liter for 22 h at 20°C) led to a 5-log reduction of the same two pathogens *(9).* Neither of these gas phase treatments adversely affects seed germination. Direct comparisons of aqueous antimicrobial agents with gaseous antimicrobial treatments involving naturally contaminated mung bean seed should be made if and when such seed becomes available for investigation.

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